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Resistance of *Pseudomonas* to Quaternary Ammonium Compounds

II. Cross-Resistance Characteristics of a Mutant of *Pseudomonas aeruginosa*

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Tube dilution experiments showed that benzalkonium chloride (BC)-resistant mutants of *Pseudomonas aeruginosa* grown in the presence of 1,000 μg of BC per ml were at least 20 times more sensitive to polymyxin B and colistin sulfate than the BC-sensitive (BCS) parent strain. BCS cells selected for resistance to 500 μg of polymyxin B per ml remained sensitive to BC. There was little difference in the amount of carbenicillin, gentamicin sulfate, or rifampin needed to prevent growth of either the BCS or BC-resistant (BCR) strains. Growth of BCR cells was inhibited by ethylenediaminetetraacetate at a concentration of 400 μg/ml or less, whereas the BCS strain grew at ethylenediaminetetraacetate levels of 10,000 μg/ml. Phenylmercuric acetate and thimerosal inhibited growth of BCR and BCS cells at concentrations of 10 μg/ml or less. BCR cells were cross-resistant to >1,000 μg/ml concentrations of five other quaternary ammonium compounds, including three with C_{16} alkyls and two with alkyl groups of shorter length. The BCS strain was also resistant to >1,000 μg/ml concentrations of the three quaternary ammonium compounds with C_{16} alkyl groups but, in addition to BC, was inhibited by 200 μg/ml levels or less of the two quaternary ammonium compounds containing alkyl groups of less than 16 carbon atoms.

Pseudomonas species resistant to quaternary ammonium compounds (QAC) have been the cause of serious infections when contaminated QAC solutions have been used either to disinfect thermolabile materials (10, 12, 14, 17, 20, 21) or as skin and wound disinfectants (2). QAC are also widely used as antimicrobial agents in many drugs in which bacterial contamination would cause a health hazard.

Substances containing quaternary nitrogens are not considered to be metabolically specific in their antibacterial action. Their bactericidal effect at low levels (100 μg/ml) has been attributed to a general dissolution of the cell membrane followed by an inactivation of cytoplasmic enzymes as concentrations of the agents increase (11, 13).

Evidence has been found that links resistance to QAC in gram-negative bacteria, including *P. aeruginosa*, to an increase in lipid, presumably at the cell envelope level (6, 15; Anderes, Ph. D. Thesis, Oregon State Univ., 1969). Treatment of QAC-resistant *P. aeruginosa* with ethylenediaminetetraacetate (EDTA) renders the cells sensitive to QAC (15). At least one action of EDTA on *P. aeruginosa* was a reported extraction of lipopolysaccharide (LPS) from the cell wall (7).

Changes in bacterial cell envelope lipid composition are also associated with resistance of gram-negative bacteria to polymyxin B and the tetracyclines (8, 18). Ampicillin resistance in mutants of *Escherichia coli* was shown to be related to a modification of LPS resulting in decreased cellular permeability and a subsequent increase in resistance to chloramphenicol, kanamycin, and streptomycin (19).

We reported earlier (1) on the ability of a mutant of *P. aeruginosa* to survive and grow in solutions containing 1,000 μg of commercial benzalkonium chloride (BC) per ml. BC is a mixed alkyl QAC widely used either as a disinfectant or to prevent bacterial contamination of drugs. Since bacterial resistance to QAC may be based on a biochemical modification of the cell envelope, cellular permeability properties could be altered. In view of the serious infections caused by QAC-resistant *Pseudomonas* and the role of QAC as a drug-preserving agent, it was of interest to examine the cross-resistance characteristics of the BC-resistant (BCR) mutant of *P. aeruginosa* to several antibiotics useful in the chemotherapy of *Pseudomonas* infections, other QAC, organic mercurials, and EDTA.

MATERIALS AND METHODS

Organism. *P. aeruginosa* was ATCC strain 9027. The bacterium was originally sensitive to BC levels above 200 μg/ml. Mutants resistant to BC levels of 36,000 μg/ml were selected as described earlier (1). Unless stated otherwise, BCR cells were grown in the presence of BC at a final concentration of 1,000 μg/ml. BCS refers to the BC-sensitive parent strain.

Medium and growth. Both the BCS and BCR strains were cultured in a sterile salts, glucose, Casamino Acids (Difco) medium containing the following per liter of triple distilled water: $CaCl_2 \cdot 2H_2O$, 0.005 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; KCl, 2.23 g; KH_2PO_4, 7.42 g; NaCl, 3.31 g; Na_2HPO_4, 2.58 g; D-glucose, 0.5% (w/v); and Casamino Acids, 0.5% (w/v). BC was membrane-filtered (Millipore, 0.22-μm porosity) and was added as required at a final concentration of 1,000 μg/ml. The final *p*H of the medium was adjusted to 7.2 with 2 N NaOH. Cells were grown stationarily at 30 C in 750-ml Erlenmeyer flasks containing 250 ml of medium. The flasks were not mechanically shaken because of the formation of large clumps in the BCR cultures. These clumps could not be readily broken to form homogeneous cell suspensions.

Susceptibility tests. Tube dilution experiments were carried out with carbenicillin, colistin sulfate, EDTA (tetrasodium salt), gentamicin sulfate, phenylmercuric acetate, polymyxin B, various QAC, rifampin, and thimerosal. Each test tube (13 by 150 mm) contained salts, glucose, Casamino Acids medium, and the test compound. Inocula were prepared from 24-hr growths of the BCS strain and 72-hr growths of the BCR strain. Both strains were in the early stationary phase of growth. The cells were washed three times and resuspended in triple distilled water. The cell suspension was adjusted so that approximately 2.5×10^6 cells were added to each test tube in a volume of 0.05 ml. The final volume in each tube was 10 ml. Antibiotics and mercurials were tested at some or all of the following concentrations (μg/ml): 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 125, 150, and 200. EDTA and QAC were tested at the following concentrations (μg/ml): 10, 50, 100, 200, 400, 600, 800, and 1,000. EDTA was tested at additional levels of 2,500, 5,000, and 10,000 μg/ml. Antimicrobial agents were made up in glucose, Casamino Acids, salts medium at *p*H 7.2 and sterilized by membrane filtration. The lowest concentration of a compound in which no growth was observed after the designated incubation period was considered as the minimal inhibitory concentration (MIC). Tubes were mixed once every 24 hr for a few seconds on a mechanical rotary mixer.

QAC. BC was *n*-alkyldimethylbenzylammonium chloride. It contained the following percentages of mixed alkyl groups: C_{12}, 50%; C_{14}, 30%; C_{16}, 17%; and C_{18}, 3%. *N*-alkyldimethyl 3,4-dichlorobenzylammonium chloride had the same percentages of mixed alkyl groups as BC. The other compounds tested [cetyldimethylethylammonium bromide (CDME), cetylpyridinium chloride, cetyltrimethylammonium bromide, and dodecyldimethyl-(2-phenoxyethyl)ammonium bromide] had single alkyl groups. All structures are shown in Table 3.

RESULTS

The data summarized in Table 1 indicate an apparent lack of cross-resistance of BC-grown BCR cells to polymyxin B and colistin sulfate.

TABLE 1. *Effect of antibacterial agents on benzalkonium chloride (BC)-sensitive and BC-resistant Pseudomonas aeruginosa*[a]

Antibacterial agent	MIC (μg/ml)											
	BC-sensitive cells				BC-resistant cells[b]							
					BC not added				BC added[d]			
	2 days[c]	5 days	7 days	10 days	2 days	5 days	7 days	10 days	2 days	5 days	7 days	10 days
Colistin sulfate	100	>100[e]			5	5	5	5	0.5	2	2	2
Polymyxin B	125	>125			6	6	6	6	1	1	1	1
EDTA	>10,000				10	200	400	400	10	100	100	100
Carbenicillin	200	>200			150	150	150	150				
Gentamicin sulfate	20	50	50	50	20	20	20	20				
Rifampin	10	20	20	20	10	10	20	20				
Phenylmercuric acetate	1	10	10	10	1	1	1	1				
Thimerosal	1	10	10	10	1	1	1	1				

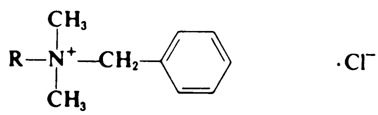
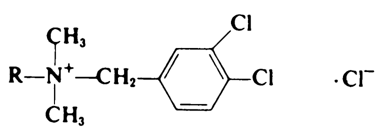
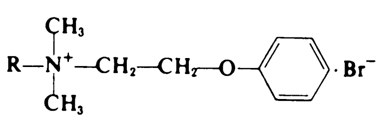
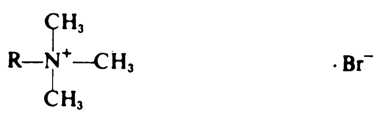
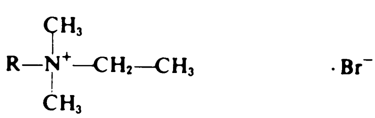
[a] Abbreviations: MIC, minimal inhibitory concentration; EDTA, ethylenediaminetetraacetate.
[b] BCR cells grown in the presence of 1,000 μg of BC per ml.
[c] Of incubation.
[d] BC (1,000 μg/ml) present in addition to the antimicrobial agent.
[e] Once an organism grew in the highest concentration of a substance tested as indicated by > preceding the MIC, that MIC is no longer recorded in the table under subsequent incubation periods.

TABLE 2. *Comparison of cross-resistance characteristics of benzalkonium chloride (BC)-resistant (grown with and without 1,000 μg of BC per ml) and polymyxin B-resistant Pseudomonas aeruginosa*[a]

Antibacterial agent	MIC (μg/ml)															
	BC-sensitive cells[b]				BC-resistant cells[b]				BC-resistant cells (grown without BC for 16 hr)				Polymyxin B-resistant cells			
	2 days[c]	5 days	7 days	10 days	2 days	5 days	7 days	10 days	2 days	5 days	7 days	10 days	2 days	5 days	7 days	10 days
BC.	200	200	200	200	>1,000				>1,000				100	100	100	100
Polymyxin B. . .	125	>125[d]			6	6	6	6	125	>125			>500			
EDTA.	>10,000				10	200	400	400	>10,000							

[a] Abbreviations: MIC, minimal inhibitory concentration; EDTA, ethylenediaminetetraacetate.
[b] Data taken partially from Table 1.
[c] Of incubation.
[d] Once an organism grew in the highest concentration of a substance tested as indicated by > preceding the MIC, that MIC s no longer recorded in the table under subsequent incubation periods.

TABLE 3. *Cross-resistance of benzalkonium chloride (BC)-sensitive and BC-resistant Pseudomonas aeruginosa to quaternary ammonium compounds*

Compound			MIC (μg/ml)[a]	
Name	R (saturated alkyl)	Quaternary nitrogen configuration	BC-sensitive	BC-resistant
Alkyldimethylbenzylammonium chloride (BC)	Mixture: C_{12} to C_{18}		100	>1,000
Alkyldimethyl 3,4-dichlorobenzylammonium chloride	Mixture: C_{12} to C_{18}		200	>1,000
Dodecyldimethyl-(2-phenoxyethyl) ammonium bromide	C_{12}		50	>1,000
Cetyltrimethylammonium bromide	C_{16}		>1,000	>1,000
Cetyldimethylethylammonium bromide	C_{16}		>1,000	>1,000
Cetylpyridinium chloride	C_{16}		>1,000	>1,000

[a] Minimal inhibitory concentration after 10 days of incubation.

Both of these antibiotics are cyclic peptides which exert their action at the cell envelope level (17). In fact, the BCR cells were at least 20 times more susceptible to these compounds than the BCS parent strain. The BCR strain was even slightly more sensitive to the antibiotics when BC (1,000 μg/ml) was present in the reaction mixture (Table 1). When BCR cells were grown in BC-free medium for 16 hr, they were found to have the same level of polymyxin B resistance as the BCS strain. These cells still retained their BC resistance (Table 2).

The BCS strain, with its relatively high level of natural resistance to polymyxin B and colistin sulfate (Table 1), frequently exhibited resistant colonies when grown on gradient plates containing up to 600 μg of polymyxin B per ml. Accordingly, it was not difficult to select BCS mutants resistant to polymyxin B at a concentration of 500 μg/ml. This was done in the manner described elsewhere for the selection of BCR cells (1). It was found that washed BCS cells that had been grown in the presence of 500 μg of polymyxin B per ml were sensitive to BC levels of 100 μg/ml (Table 2).

Analogous to colistin sulfate and polymyxin B, there was a striking difference in the sensitivity to EDTA between the BCR (grown in the presence of BC) and BCS strains (Table 1). Although the BCS cells grew in the presence of 10,000 μg of EDTA per ml (the highest level tested), 400 μg (MIC at 10 days) of this substance per ml prevented the growth of the BCR strain (Table 1). When 1,000 μg of BC per ml was added, the MIC for BCR cells was 100 μg of EDTA per ml after 10 days. The acute EDTA sensitivity of the BCR strain was lost when the cells were grown in the absence of BC for 16 hr. Table 2 shows that these cells reverted to an EDTA resistance similar to that of the BCS strain.

In contrast to polymyxin B, colistin sulfate, and EDTA, there appeared to be no significant difference in the level of carbenicillin needed to suppress growth of the BCS and BCR cells after a 48-hr incubation period (Table 1). The same was true for gentamicin sulfate and rifampin. The organic mercurials, phenyl mercuric acetate and thimerosal, also had high degrees of activity against both the BCS and BCR strains (Table 1).

Examination of the BCS and BCR strains for cross-resistance to other QAC led to the results presented in Table 3. In addition to BC, the BCR strain was resistant to >1,000 μg/ml levels of all five compounds tested. The BCS strain was found to be naturally resistant to >1,000 μg/ml levels of cetylpyridinium chloride, CDME, and cetyltrimethylammonium bromide. These compounds are all characterized by a C_{16} alkyl group. On the other hand, the BCS strain was sensitive to dodecyl-dimethyl-(2-phenoxyethyl) ammonium bromide, which contains a C_{12} alkyl group and alkyldimethyl 3,4-dichlorobenzylammonium chloride. The last is a chlorinated form of BC and, as with BC, 50% of the molecules have C_{12} alkyl groups.

To determine if the natural resistance of the BCS strain to a QAC with a C_{16} alkyl group could be overcome by the presence of EDTA, a

TABLE 4. *Effect of cetyldimethylethylammonium bromide (CDME) and ethylenediaminetetraacetate (EDTA) on benzalkonium chloride (BC)-sensitive Pseudomonas aeruginosa*

Compound	MIC[a] (μg/ml)			
	2 days)[b]	5 days	7 days	10 days
CDME	>1,000[c]			
EDTA	>10,000			
EDTA in the presence of 1,000 μg of CDME per ml	100	100	200	200

[a] Minimal inhibitory concentration.

[b] Of incubation.

[c] Once an organism grew in the highest concentration of a substance tested as indicated by > preceding the MIC, that MIC is no longer recorded in the table under subsequent incubation periods.

tube dilution experiment was set up with increasing concentration of EDTA and a constant (1,000 μg/ml) level of CDME. It was found that, after 10 days of incubation, the MIC of the EDTA-CDME combination was 200 μg of EDTA per ml (Table 4). Therefore, it appears that EDTA acted synergistically with CDME against the BCS strain.

DISCUSSION

The results presented here indicate that BCR cells of *P. aeruginosa* grown in the presence of BC had markedly enhanced sensitivities to compounds that react with the cell envelope, as evidenced by the comparative inhibition data obtained with colistin sulfate, polymyxin B, and EDTA.

In the case of the antibiotics polymyxin B and colistin sulfate, the increased susceptibility of the BCR strain could be interpreted simply on the basis of a potentiating or synergistic effect on the action of BC. It is known that QAC can bind strongly or even irreversibly to the cell walls of bacteria and are not easily removed by washing (6, 16; Adair, Geftic, Gelzer, *unpublished data*). Thus, when BCR cells covered by a layer of BC molecules were placed into a solution containing polymyxin B or colistin sulfate, the organisms may essentially have been exposed to BC and the antibiotics simultaneously.

Some support for the synergistic effect of cell envelope-active antibiotics and cell-bound BC molecules comes from our finding that, when BCR cells were cultured in the absence of BC for 16 hr, they had the same low level of polymyxin B sensitivity as the BCS strain while retaining resistance to BC. During growth in BC-free medium, cell-bound BC molecules would be "diluted" by cell division. Therefore, BC molecules would no longer be present on the cell walls of BCR cells in great enough concentration to react synergistically with other cell envelope-active agents.

The finding that BCS cells selected for resistance to 500 μg of polymyxin B per ml still remained sensitive to low levels of BC is compatible with data of other investigators and suggests the existence of two distinct mechanisms of resistance to polymyxin B and QAC. Newton (18) and Brown and Watkins (5) found polymyxin B resistance in *P. aeruginosa* to be associated with a decreased number of negatively charged lipid phosphate groups in the cell envelope. The phosphate groups were thought to be sites of polymyxin B attachment. Conversely, Anderes (Ph.D. Thesis, Oregon State Univ., 1969) reported mutants of *P. aeruginosa* grown in the presence of QAC to contain 8% more bound and 9.5% more free phospholipid than either the QAC-sensitive parent strain or resistant cells grown in the absence of QAC. The differences in lipid phosphate concentrations between polymyxin B and QAC-resistant cells may offer an alternative explanation other than synergism for the apparent increased sensitivity of the BC-grown BCR strain to polymyxin B in that BCR cells with an increased number of negative phosphate groups might be more sensitive to polymyxin B. However, this explanation suffers when it is considered that, with BC-grown BCR cells, negatively charged cell envelope sites might be occupied to a great extent by positively charged BC molecules and thus not be available for reaction with polymyxin B. Indeed, it has been shown by electrophoretic mobility studies that QAC-resistant cells lose their electronegativity due to the absorption of QAC molecules to the cell surface (6, 16).

The parent strain of *P. aeruginosa* used in this study has an unusual degree of resistance to EDTA, whereas the BC-grown BCR mutant was highly sensitive to EDTA. As with polymyxin B and colistin sulfate, the sensitivity of BCR cells to EDTA may be attributed to synergism between EDTA and cell-bound BC. BCR cells grown in the absence of BC for 16 hr reverted to an EDTA resistance similar to the BCS strain. The loss of EDTA sensitivity in the absence of BC might (as with polymyxin B) be attributed to a physical dilution of cell wall-attached BC molecules based on cell division. MacGregor and Elliker (15) showed QAC-resistant mutants of *P. aeruginosa* to be killed by combinations of EDTA and QAC over a period of 300 sec. EDTA is also known to enhance greatly the action of polymyxin B and QAC against QAC-sensitive cells of *P. aeruginosa*, probably by chelating structurally essential cell hull cations with a concomitant loss of LPS and increase in cellular permeability (3, 4, 9). In view of the serious nosocomial infections that have resulted from *Pseudomonas* growth in QAC solutions (2, 10, 12, 14, 17, 20, 21), it is essential that precautions be taken to prevent growth of accidentally introduced resistant mutants if QAC are to continue to be used as disinfectants and as antimicrobial agents in drugs. Our data indicate that the combination of EDTA and QAC may offer a solution to this problem. However, it should be emphasized that BCR cells will definitely not grow in BC solutions if the only carbon and nitrogen source is the BC molecule itself (1). Thus, a sterile solution composed of ammonium acetate-free BC and distilled water will not support bacterial growth unless exogenous carbon and nitrogen are accidentally added.

No significant variance in the amount of carbenicillin needed to inhibit the growth of either the BSC or BCR strain was observed after 48 hr of incubation. The same held true for gentamicin sulfate and rifampin. Therefore, it appears that the permeability of BCR cells was not altered as regards these antibiotics. The fact that the BCR strain did not increase in resistance to any of the antibiotics tested is important from a chemotherapeutic standpoint since carbenicillin and gentamicin sulfate, along with polymyxin B and colistimethate, are the drugs most effective against the majority of *Pseudomonas* infections. Fortunately, initial observations on the pathogenicity of BCR cells have shown this organism to be at least 10 times less pathogenic than the BCS parent strain (Liauw et al., Bacteriol. Proc., p. 104, 1970).

In this study, it was found that the BCS parent strain of *P. aeruginosa* was naturally resistant to three QAC, each of which contained a C_{16} alkyl group, but differed with respect to other quaternary nitrogen-bound groups. Thus, it appears that substitutions in the QAC molecule, apart from the alkyl chain, do not readily contribute to the activity of these compounds. A combination of EDTA and one of the C_{16} alkyl compounds (CDME) resulted in a synergistic effect that inhibited growth of the BCS strain. This result again serves to emphasize the value of EDTA-QAC combinations. Other than BC, the BCS strain was sensitive to QAC containing C_{12} alkyl groups. From a practical standpoint, these data suggest that, as an alternative to the addition of EDTA, problems of bacterial resistance to QAC with C_{16} alkyl chains (2) may be overcome by selecting a QAC with a C_{12} alkyl chain.

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